GENE LINKAGE IN DNA TRANSFER: A CLUSTER OF GENES CONCERNED WITH AROMATIC BIOSYNTHESIS IN BACILLUS SUBTILIS[1]

EUGENE W. NESTER,[2] MARION SCHAFER,[3] AND JOSHUA LEDERBERG

Department of Genetics, Stanford University School of Medicine, Palo Alto, California

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INITIAL studies on the structure of the segmental genetic map in *Bacillus subtilis* have revealed a close linkage relationship between genes of indole and histidine biosynthesis (EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; NESTER and LEDERBERG 1961). More recently, ANAGNOSTOPOULOS and CRAWFORD (1961) showed that this same molecule of DNA carries at least four of the loci for tryptophan biosynthesis, from anthranilic acid to tryptophan, with the linkage and biosynthetic pathway following the same sequential order with one possible exception. In contrast, the histidine loci are dispersed among two or more linkage groups. The present studies extend the genetic map of this cluster of markers of aromatic amino acid biosynthesis. Additional mutants requiring one or more "aromatic metabolites"[4] such as phenylalanine, tyrosine, tryptophan or shikimic acid have been isolated and given a preliminary biochemical characterization. Many of these mutants are also linked to the try_2 segment.

Studies on the enteric bacteria have revealed a remarkable pattern of linkage of genes controlling the biosynthesis of enzymes of related biosynthetic functions (DEMEREC and HARTMAN 1959). In several instances, the genetic map order nearly parallels the enzymatic sequence (HARTMAN, LOPER and SERMAN 1960; YANOFSKY and LENNOX 1959). In Neurospora, there is at least one gene cluster which likewise involves the aromatic metabolites (GROSS and FEIN 1960). The clustering of genes with related functions to form an "operon" is believed to reflect the regulatory mechanisms by which these functions are coordinated (JACOB and MONOD 1961).

MATERIALS AND METHODS

Media: Four basic media were employed: Difco antibiotic medium 3 (A3)—a complete medium (liquid). Modified Davis minimal (D) (LEDERBERG 1950)—

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[2] Present address: Department of Microbiology, University of Washington School of Medicine, Seattle, Washington.

[3] Present address: Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia.

[4] i.e., compounds containing a 6-carbon ring related to the metabolism of aromatic amino acids: phenylalanine, tyrosine, tryptophan, or p-aminobenzoic acid.

the basal medium for all platings. It was routinely supplemented with DL-glutamic acid (10 μg per ml) and L-asparagine (10 μg per ml). Spizizen minimal (S) (SPIZIZEN 1958)—the basal medium in preparing competent cells. Nutrient Agar (Difco) (N.A.).

Bacterial strains: (Tables 1 and 2). All strains were derived from *Bacillus subtilis* 168 try_2^- and 23 thr^- (BURKHOLDER and GILES 1947). In most of the mapping experiments, triply auxotrophic strains were employed as recipients. These strains were prepared by the following transformation procedure. DNA, extracted from the strain carrying the desired *aro* marker, was used to transform a triply marked strain try_2^- his_2^- leu_1^- (SB 98). Transformants were plated on specifically supplemented media and screened for the *aro* requirement which is sometimes introduced by random coincidence (one to two percent).

In this laboratory, mutants are routinely obtained by the following procedure: Cells are grown for 18 hours in A3 medium to the stationary phase, washed once, and diluted to a cell concentration of approximately 2×10^8 cells per ml in D medium. The cells are irradiated with a low pressure mercury ultraviolet lamp to a survival of 10^{-4}, and incubated for four to 18 hours in minimal medium with appropriate supplements to allow growth of specific mutants. The culture is then washed, diluted 1:10 in minimal medium and starved for 60 minutes. Two thousand units of penicillin per ml are added, and incubation continued for four hours. Two thousand units of penicillinase per ml are then added, incubation continued for an additional 15 minutes, after which the culture is plated on N.A. The surviving colonies are tested by replica plating to supplemented minimal agar and sometimes yield ten to 20 percent *aro* mutants.

Transformation procedure: Recipient cells are routinely brought to a state of competence by the following regimen. Cells are grown for approximately 12 hours in A3 medium, washed and resuspended in S medium containing 0.3 percent yeast extract. After four hours incubation, the cells are again washed and diluted 1:10 into S medium containing 0.1 percent yeast extract. DNA is added 90 minutes later, followed by deoxyribonuclease (20 μg per ml in 0.01 M $MgCl_2$, final concentration) after an additional 36 minutes. The detailed conditions of incubation and assay have been described previously (NESTER and LEDERBERG 1961). The use of yeast extract was desirable because of the variety of auxotrophic strains employed in the course of the present investigation. It resulted in a competent population of all mutants on which it was used. However, at saturating levels of DNA, transformation rates rarely exceeded 0.05 percent. In more recent experiments, specifically noted, the casein hydrolysate regimen was employed and CHT-2 (0.02 percent acid-hydrolyzed casein and 20 μg per ml of DL-tryptophan) and CHT-10 (0.1 percent acid-hydrolyzed casein and 50 μg per ml of DL-tryptophan) were each fortified by the addition of tyrosine, phenylalanine, tryptophan, p-aminobenzoic acid, and p-hydroxybenzoic acid at concentrations listed in Table 2. With this procedure, transformation rates of 0.05 to 0.2 percent for most of the *aro*-metabolite mutants were routinely obtained when saturating levels of DNA were employed. The two regimens gave comparable linkage patterns. Similarly, a marked improvement in trans-

TABLE 1

List of strains of Bacillus subtilis *and* Escherichia coli

Strain number	Genotype*	Origin
23	*thr*$^-$	BURKHOLDER and GILES 1947
168	*try*$_2^-$	BURKHOLDER and GILES 1947
SB 19	Reference prototroph; *str*^{r}	23—× 168†; *str* selection
SB 25	*try*$_2^-$ *his*$_2^-$	UV treatment of strain 168
SB 29	*try*$_2^-$ *tyr*$_1^-$	UV treatment of 168
SB 30	*try*$_2^-$ *tyr*$_2^-$	UV treatment of 168
SB 32	*his*$_2^-$	SB 19 —× SB 25
SB 33	*try*$_2^-$	SB 19 —× SB 25
SB 65	*tyr*$_1^-$	SB 19 —× SB 29
SB 70	*his*$_2^-$ *tyr*$_1^-$	SB 65 —× SB 25
SB 98	*try*$_2^-$ *his*$_2^-$ *leu*$^-$	Spontaneous mutation in SB 25
SB 100	*try*$_2^-$ *his*$_2^-$ *tyr*$_1^-$	SB 70 —× SB 98
SB 103	*tyr*$_1^-$	SB 19 —× SB 70
SB 112	*try*$_2^-$ *phe*$_1^-$	UV treatment of 168
SB 116	*try*$_2^-$ *aro*$_5^-$	UV treatment of 168
SB 125	*his*$_2^-$ *inh*$_1^-$	UV treatment of SB 32
SB 126	*his*$_2^-$ *inh*$_2^-$	UV treatment of SB 32
SB 128	*his*$_2^-$ *inh*$_3^-$	UV treatment of SB 32
SB 130	*try*$_2^-$ *aro*$_1^-$	UV treatment of 168
SB 133	*phe*$_1^-$	SB 19 —× SB 112
SB 136	*his*$_2^-$ *aro*$_4^-$	UV treatment of SB 32
SB 137	*his*$_2^-$ *aro*$_2^-$	UV treatment of SB 32
SB 148	*his*$_2^-$ *aro*$_3^-$	UV treatment of SB 32
SB 181	*try*$_2^-$ *aro*$_2^-$	SB 33 —× SB 137
SB 182	*try*$_2^-$ *his*$_2^-$ *aro*$_1^-$	SB 130 —× SB 98
SB 188	*aro*$_1^-$	SB 33 —× SB 130
SB 190	*try*$_2^-$ *his*$_2^-$ *aro*$_2^-$	SB 181 —× SB 98
SB 194	*try*$_8^-$	DR. C. ANAGNOSTOPOULOS, Western Reserve University
SB 200	*try*$_8^-$ *his*$_2^-$ *aro*$_2^-$	SB 194 —× SB 190
SB 202	*try*$_2^-$ *his*$_2^-$ *tyr*$_1^-$ *aro*$_2^-$	SB 181 —× SB 100
SB 224	*try*$_2^-$ *his*$_2^-$ *aro*$_3^-$	SB 148 —× SB 98
SB 235	*try*$_2^-$ *his*$_2^-$ *aro*$_5^-$	SB 116 —× SB 98
SB 253	*his*$_2^-$ *aro*$_5^-$	SB 19 —× SB 235
SB 254	*mtr*^{s} *aro*$_3^-$ *tyr*$_1^-$	SB 103 —× SB 148
SB 401	*aro*$_1^-$ *aro*$_3^-$	SB 188 —× SB 148
SB 419	*inh*$_1^-$	SB 33 —× SB 125
SB 426	*mtr*^{r}	Spontaneous mutation SB 19
SB 428	*suh*$^-$	Spontaneous mutation SB 32
SB 443	*inh*$_3^-$	SB 33 —× SB 128
SB 454	*inh*$_2^-$	SB 168 —× SB 126
SB 474	*try*$_2^-$ *mtr*^{r}	SB 426 —× SB 181
E. coli–170–143S1	*aro*$^-$	DR. B. DAVIS
E. coli–83–1	*aro*$^-$	DR. B. DAVIS
E. coli–W7 (58–278)	*phe*$^-$ *bio*$^-$	TATUM 1945

* A completely rational system for the nomenclature of mutants is hard to devise with the flux of knowledge and the conflict of precedence. For the present, in line with the suggestions of DEMEREC (1956), mutants requiring a single amino acid will be designated by the abbreviation for it—*viz.*, *his*, *phe*, *tyr*, *try*, with a subscript number indicating the order of isolation. Abbreviations will follow the suggestions of the Editors of the *Journal of Biological Chemistry*. The interrelationships in aromatic biosynthesis complicate the terminology. We propose the class name "aromatic", abbreviated *arom* for the entire group which will comprise (a) specific auxotrophs—e.g., *phe*$_1^-$ *tyr*$_2^-$ *try*$_2^-$—and (b) mutants which require more than one end product—e.g., *aro*$_2$, *aro*$_3$, etc. Under this system, strain 168, *ind*$^-$ will be referred to as *try*$_2^-$. This provisional nomenclature will be superseded when the enzymology of the entire pathway is worked out and each mutant in the system can be designated by its particular enzymatic alteration.

† The symbol 23 —× 168 indicates that DNA from strain 23 was used to transform the recipient strain 168.

TABLE 2

Nutritional response of listed mutants

Symbol	Normal growth factor(s)*	Additional responses
thr	threonine	.....
try_2	tryptophan	indole
his_2	histidine	histidinol†
leu	leucine + isoleucine	.....
tyr_1	tyrosine	p-hydroxyphenylpyruvic acid
aro_1 aro_5	tyrosine + phenylalanine + tryptophan + para-amino benzoic acid	.....
aro_2 aro_3 aro_4	tyrosine + phenylalanine + tryptophan + para-amino benzoic acid‡	shikimic acid
try_8	tryptophan	indole, anthranilic acid
suh_1	histidine	phenylalanine
phe_1	phenylalanine	phenylpyruvic acid

* The concentration of the growth factors in liquid medium in μg per ml: anthranilic acid–10, DL-phenylalanine–20, L-histidine–10, indole–10, L-isoleucine–10, L-leucine–10, para-aminobenzoic acid–0.01, shikimic acid–25, L-threonine–20, L-tryptophan–10, L-tyrosine–10.

† In our hands, SB 32 responds poorly to chromatographically pure histidinol.

‡ Depending on their degree of leakiness, multiple aromatic mutants may require less than the total complement of aromatic compounds for normal growth.

formation frequency was often found for other auxotrophic mutants when the specific requirements (10 μg per ml) of the recipient cells were added to CHT-1 and CHT-10 in place of yeast extract.

DNA was prepared as previously described (NESTER and LEDERBERG 1961). In all mapping studies of the aromatic mutants, DNA was added at levels corresponding to the linear portion of the dose-response curve, 0.01 to 0.03 μg per ml of DNA with 2 to 6 $\times$ 10^8 recipient cells.

Scoring of genotypes: The following procedure was employed in scoring transformant genotypes resulting from the cross of wild-type DNA with a triply auxotrophic recipient strain. The transformed recipient population was plated on three different sets of minimal media, each supplemented with two of the growth requirements, thereby making one of the three auxotrophic recipient markers selective. At least two-hundred widely separated colonies of each primary transformant type were then stroked onto nutrient agar plates (50 per plate) with sterile toothpicks. The remainder of the genotype of each colony was then determined by its growth response after replica plating to sets of singly supplemented media.

Some genotypes might be incorrectly scored by the procedure just summarized. For example, in Table 13, a mixed colony of type 100 (++−) plus type 011 (−−+) would be scored as a type 101 (+++). Such admixture may arise from segregation of markers within a single clone or from trivial accidents. This source of error can be controlled by preliminary purification of the clones, and by replica plating on minimal as well as doubly-supplemented medium in scor-

ing transformant clones. The occurrence of such confusions has never involved more than five percent of the clones. They would not seriously influence the interpretation of the results except in the combination $a^+b^-c^+ \text{——} \times a^-b^+c^-$ in which the prototroph constitutes the 101 class. In this experiment (Table 13) special care was taken to use both of the indicated cautions. In all experiments, replica plating onto minimal media was routinely done.

Identification of biochemical phenotypes: The pathway of aromatic amino acid biosynthesis (Figure 1) is known mainly from nutritional and enzymatic studies with *E. coli* (DAVIS 1955; SPRINSON 1960; RIVERA and SRINIVASAN 1962; MORGAN, GIBSON and GIBSON 1962). Current studies are aimed at assigning a specific locus for each of the enzymatic blocks, especially by the identification of accumulated intermediates and enzymatic studies on mutant strains. For the purpose of accumulating intermediates, cultures were grown aerobically at 37° for 24 to 48 hours in S medium supplemented with the five aromatic supplements (see Table 2). The culture supernates were sterilized by filtration (Millipore HF) and analyzed by bioassay and bioautography on *E. coli* 83-1 (*aro*$^-$) kindly provided by DR. B. DAVIS, using the procedures described by DAVIS and MINGIOLI (1952). Supernates were chromatographed on Whatman 1 filter paper, using a butanol-formic acid solvent (n-butanol, 50 ml; formic acid, 2.5 ml; H_2O, 10 ml).

Chemical assays: DNA was determined by the procedure of BURTON (1956) using deoxyadenylic acid as a standard.

The accumulation of prephenic acid was deduced from its conversion to phenylpyruvic acid at a low pH, as described by METZENBERG and MITCHELL (1956). The ultraviolet spectrum of the conversion product was compared with an authentic sample of phenylpyruvic acid, employing a Beckman DK-2 Recording Spectrophotometer.

Enzyme assays: Tryptophan synthetase activity of intact cell suspensions was assayed by the procedure of EISENSTEIN and YANOFSKY (1962).

Amino acid analysis of radioactivity: The protein was isolated by the procedure of ROBERTS, ABELSON, COWIE, BOLTON and BRITTEN (1955), and hydrolyzed in twice distilled 5.7 N HCl in sealed, evacuated tubes at 105°C for 24 hours. Amino acid analyses were carried out with a Spinco amino acid analyzer, and the radioactivity assayed by a Packard Tri-Carb Flow Monitor.

Chemicals: Metabolites including the radioactive compounds were generally purchased from California Corporation for Biochemical Research. 5-methyl tryptophan and histidinol were purchased from Cyclo Chemical Co. The latter compound was purified by paper chromatography; the rest were used without further purification. Deoxyribonuclease (1 × crystallized) was purchased from Worthington Biochemical Corporation.

RESULTS

Linkage of markers to the try_2 *cluster:* Which markers are closely linked to the loci of indole glycerol phosphate (try_2) and histidine biosynthesis (his_2)? A wide variety of mutant markers was introduced into strain 168 giving double

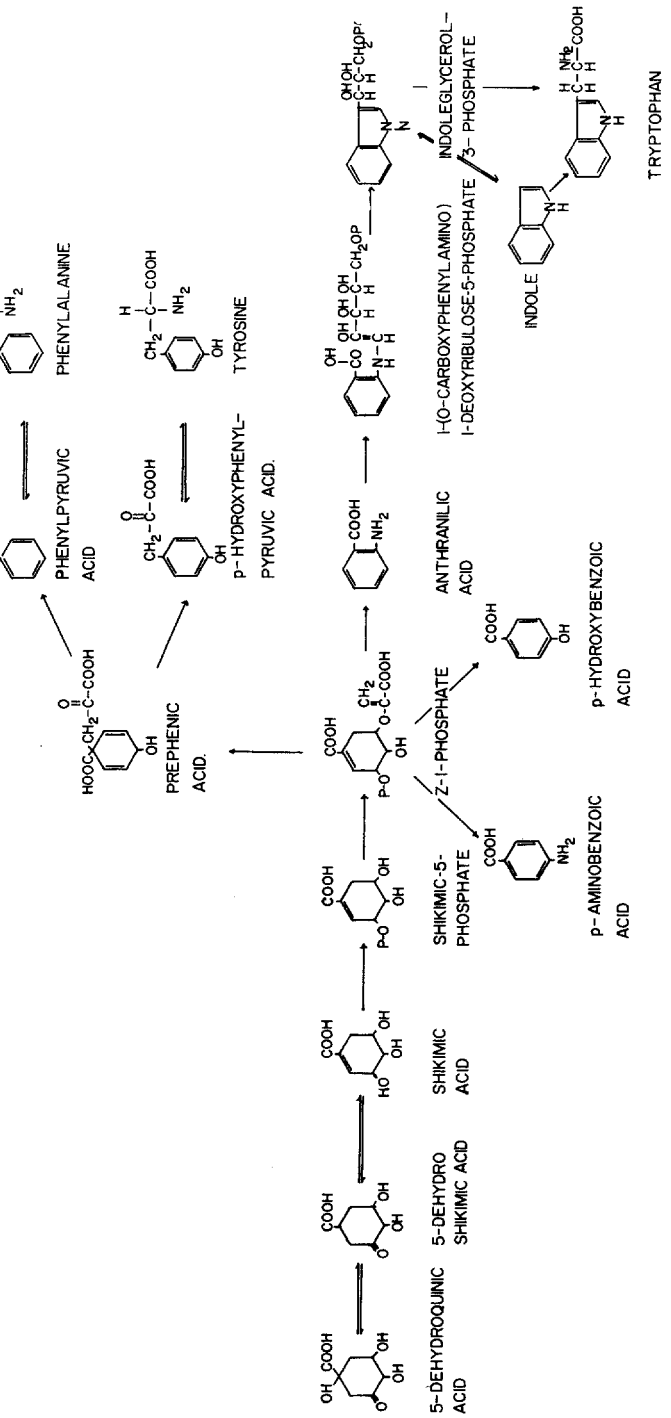

FIGURE 1.—Aromatic amino acid biosynthesis in *E. coli:* This pathway is derived mainly from the data of DAVIS 1955, SPRINSON 1960, RIVERA and SRINIVASAN 1962, and MORGAN *et al.* 1962.

auxotrophs useful for testing the linkage of try_2 to the other markers. If wild-type DNA produced a high proportion of prototrophs compared to either of the single transformant types, this was preliminary evidence for linkage of the two markers (Table 3). In some instances, notably the nitrous acid mutants, the recipient carried only one mutant locus. In these cases, linkage was tested by determining the cotransfer of the mutant locus with try_2 in a repulsion cross with 168: $X^- try_2^+ \text{ —— } \times X^+ try_2^-$.

The linkage relationship can be expressed as the cotransfer index, r, a measure of the frequency of joint transfer of two markers compared to the total number of new genotypes measured by the transformation experiment. To use a general notation (LEDERBERG 1957), in a system $11 \text{ —— } \times 00$ (a simplified representation of $a'b' \text{ —— } \times a^0b^0$, in which the donor markers are designated as 1 and the recipient markers, 0), giving transformant types 11, 10 and 01, $r = 11/(11+01+10)$.

In some experiments to be reported in this paper (Table 5), it is possible to estimate $a'b'$ and a^0b', but not $a'b^0$, the latter representing the double auxotroph genotype in a repulsion experiment, $a^-b^+ \text{ —— } \times a^+b^-$. In this case we approximate $10 = 01$ and write the index $r = 11/[11 + 2(01)]$. Of markers tested,

TABLE 3

Linkage tests on mutant B. subtilis *strains*

Genotype	Mutagen	Cotransfer index	Competency regimen*
Linked:			
$try_2^- tyr_1^-$	UV	0.41	1
$try_2^- tyr_2^-$	UV	0.35	1
Unlinked:			
$try_2^- phe_1^-$	UV	0.012	1
$try_2^- pur_1^-$	UV	0.038	2
pur_2^-	HNO_2	0.020	2
pyr_1^-	UV	0.010	2
pyr_2^-	HNO_2	0.005	3
$try_2^- gly^-$	UV	0.048	2
$try_2^- met_1^-$	UV	0.03	4
met_2^-	HNO_2	0.02	4
$try_2^- ile\text{-}leu^-$	Spontaneous	0.009	3
$try_2^- met\text{-}lys^-$	UV	0.016	4
glu^-	HNO_2	0.024	4
pro^-	HNO_2	0.018	4
$try_2^- his_1^-$	UV	0.0050	4
$try_2^- cys^-$	UV	0.0080	4
$try_2^- val\text{-}ile^-$	UV	0.011	4
$try_2^- arg^-$	Spontaneous	0.010	4
$try_2^- emb^r$	Spontaneous	0.015	4
$try_2^- str^r$	Spontaneous	0.020	4
leu^-	HNO_2	0.010	3
$his_2^- nia^-$	UV	0.009	2

* The competency regimen refers to: (1) CHT–2 and CHT–10 with aromatic supplements added; (2) Yeast extract regimen; (3) CHT–2 and CHT–10 with 10 μg per ml of the recipient strains requirement added to each medium; (4) CHT–2 and CHT–10 regimen with no supplementation.

only *tyr* (tyrosine) and try_2 are closely linked (Table 3). The observed value of r is 0.41. If the markers were unlinked, r_u would be $5/\alpha \times 10^{-5}$ for these experiments, α being the fraction of the recipient cells which are competent. For unlinked markers, cotransfer depends on random coincidence. We can approach an estimate of r_u by the limiting fraction of transferred bacteria obtained at high DNA concentrations. This figure, α, sometimes reaching .02 and usually about .001 for a given marker, varies from culture to culture and with the conditions of their growth. Furthermore, it does not reflect the total number of potentially competent cells for other markers.

The uncertainty in the estimation of r_u at lower DNA levels suggests the importance of further analysis to verify linkage, especially in any crucial test. The essential element of the linkage hypothesis is that a single molecule of DNA, indivisible by dilution, carries two or more markers. If this is true, then r should extrapolate to a finite value at infinite dilution; it should tend to zero if two separate transforming molecules must cooperate to give the co-transfer type.

This distinction is clearly shown in Figures 2 and 3, for the linkage of his_2 try_2

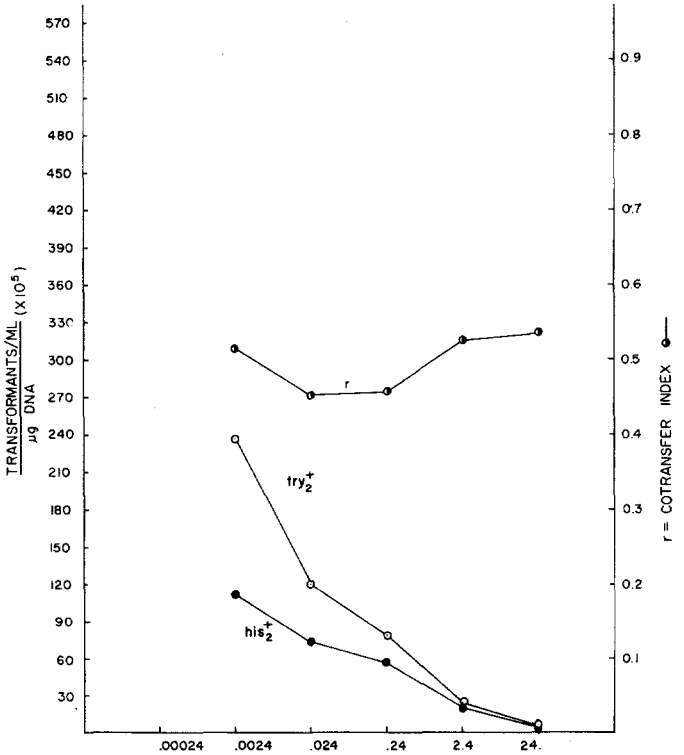

FIGURE 2.—*DNA dose/linkage kinetics of linked markers:* SB 25 was brought to competence by the CHT-2 and CHT-10 regimen. DNA was added at the indicated concentrations, and transformants plated on D + histidine and D + tryptophan. One hundred colonies from each plate, at each DNA concentration were picked to N.A. and replica plated to D. The closed and open circles are plots of apparent specific activity, the ratio of transformants found to DNA applied, and show saturation with excess DNA.

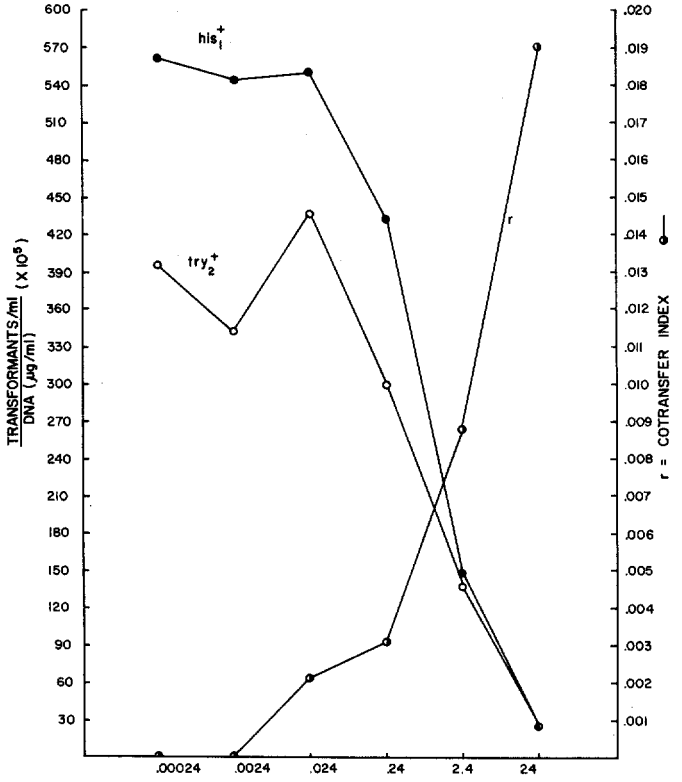

FIGURE 3.—*DNA dose/linkage kinetics of unlinked markers:* SB 1 was brought to competence by the CHT-2 and CHT-10 regimen. DNA was added at the indicated concentrations, and transformants plated on S, S + histidine, and D + tryptophan. The cotransfer data were verified by replica plating the D + histidine and S + tryptophan to D. The closed and open circles are plots of apparent specific activity, the ratio of transformants found to DNA applied, and show saturation with excess DNA.

vs. the non-linkage of his_1 try_2, r extrapolating to 0.5 *vs.* <.02. The same point is illustrated in Table 4, the DNA dose-kinetics of linkage of try_2-tyr_1.

The congression of markers from distinct molecules of transforming DNA (markers then appearing in the same transformant clone) can be tested directly by using a mixture of DNA from two donor strains. This is shown in Table 5 for $(try_2^- tyr_1^+) + (try_2^+ tyr_1^-) \longrightarrow \times try_2^- tyr_1^-$. Congression does occur as indicated by the appearance of try_2 tyr_1 prototrophs but too rarely ($r_c = 0.004$) at the DNA concentrations used to confound the estimation of linkage ($r = 0.4$).

From these considerations, the cotransfer index for linked markers should show a slight increase with DNA concentration due to congression. This is difficult to establish, however, against the background of linkage. In an occasional experiment, unusually high values of r have been seen at very high DNA levels (>10 μg per ml), the increment being more than would be expected from the congression of unlinked markers.

TABLE 4

DNA concentration vs. *cotransfer index*

Cross SB 19—× SB 29

DNA concentrations (μg per ml)	Cotransfer index, r
2.1	0.41
0.21	0.40
0.021	0.38
0.0021	0.35

The recipient culture was made competent by the yeast extract regimen. The calculation of r was determined from transformant counts on D media, D+tyrosine, and D+tryptophan.

TABLE 5

Congression of markers tyr_1 *and* try_2

Donor DNA	Recipient cells	Transformant classes	No. colonies scored/0.1 ml	Cotransfer index
try_2^+ try_1^-		try_2^+	192	.0044
—×	try_2^- tyr_1^-	tyr_1^+	258	
try_2^- tyr_1^+		try_2^+ tyr_1^+	2	
try_2^+ tyr_1^+—×	try_2^- tyr_1^-	try_2^+	563	
		try_1^+	695	0.41
		try_2^+ tyr_1^+	367	
try_2^+ tyr_1^- —×	try_2^- tyr_1^+	try_2^+ tyr_1^-	696	0.35
		try_2^+ tyr_1^+	337	
try_2^- tyr_1^+—×	try_2^+ tyr_1^-	try_2^- tyr_1^+	143	0.43
		try_2^+ tyr_1^+	71	

The recipient cells were prepared for competency by the yeast extract regimen except for strain try_2^- tyr_1^+ (168), which was prepared by the casein hydrolysate regimen. The donor DNA concentration varied from 0.01 to 0.03 μg per ml. Transformant classes were scored by plating on appropriately supplemented media.

Map distance: To express the map distance, q, between two markers we define $q = 1\text{-}r = (10 + 01)/(11 + 10 + 01)$, or the ratio of crossovers to total recognizable transformants.

Linkage tests on additional aro *loci:* Table 6 lists tests on representative aromatic mutants which were collected for this purpose. Some are indeed linked to his_2. Others, aro_4 and aro_5, display no linkage, a difference which may correspond to different enzymatic steps, although we do not yet have direct evidence on this point. Further, none of the six phenylalanine mutants are linked to his_2.

In tests among these mutants no significant linkage was detected between aro_4, aro_5 and phe_1 (Table 7).

Linkage order of loci in the try_2 *cluster:* The ordering of any three loci was achieved by three-point testcrosses, usually +++ —× ––– (prototrophic DNA with a triply marked auxotroph as the recipient.) In this cross, seven of the eight recombinational classes can be scored. Using a notation which designates the donor markers as 1 and the recipient markers as 0, these seven classes become 111, 110, 101, 100, 011, 010, 001. The remaining combination, 000, is the

TABLE 6

Cotransfer indices aro–his_2, try_2–his_2, *and* phe_1–his_2

Aromatic genotype	Cotransfer index
aro_2	0.3
aro_3	0.25
aro_4	<0.0008
tyr_1	0.77
aro_1	0.57
aro_5	0.013
try_8	0.36
try_2	0.50
phe_1	0.012

All cells were prepared for competency by the yeast extract procedure, and transformed with SB 19 DNA, at concentrations from 0.01 to 0.03 μg per ml. The single transformants were scored on singly supplemented plates, and the double transformants on minimal plates.

The cotransfer index of the linked markers was determined by three-point crosses; picking 200 transformant colonies, and determining the remainder of their genotype by replica plating onto appropriately supplemented media. The cotransfer index of all unlinked markers except his_2 phe_1 was determined by two point crosses: SB 19 —× aro^- his_2^- and counting the number of single and double transformants on appropriate media. To determine the cotransfer of his_2 phe_1, the cross: SB 32 —× SB 133 was performed and phe^+ transformants selected on D medium + histidine. 100 colonies were picked to N.A. and replicated to D, D + histidine and D + phenylalanine to determine the remainder of their genotype.

TABLE 7

Linkage of try_2 *unlinked aromatic mutants*

	Cross	Aromatic genotype	Cotransfer index
1.	SB 136 —× SB 116	aro_4^-–aro_5^-	0.010
2.	SB 133 —× SB 253	phe^-–aro_5^-	0.019
3.	SB 133 —× SB 136	phe^-–aro_4^-	<0.10

Cross 1—aro_5 transformants selected on D medium + tryptophan.

Cross 2—aro_5 transformants selected on D medium + histidine.

Cross 3—aro_4 transformants selected on D medium + phenylalanine.

100 transformants from each cross picked to N.A. and replicated to appropriately supplemented media to determine rest of the genotype.

recipient culture which, of course, predominates over any of the transformants. The transformants in each class are calculated as the percentage of the total colonies picked adjusted to the total number of transformants. In a three point cross, the quadruple crossover class, 101 is axiomatically the least frequent. Conversely, of the three possible arrangements the one which renders the 101 class the least frequent is inferred.

This experimental design was used to map the following sets of markers: try_2 his_2 tyr_1 (Table 8), aro_2 try_8 his_2 (Table 9), aro_3 try_2 his_2 (Table 10), try_2 his_2 aro_1 (Table 11).

The confusion of phenotypes hinders this use of the 101 frequency to order aro_2-aro_3 both of which respond to shikimic acid and likewise tyr_1 aro_1, both of which require tyrosine. The higher cotransfer index of aro_2-his_2 (0.31) compared to aro_3 his_2 (0.25) (Tables 9, 10 and 12) indicates that his_2 is closer to aro_2 than to aro_3. This arrangement agrees with the cotransfer values of aro_2 and aro_3 with the try_2 locus (Tables 10 and 12).

TABLE 8

Linkage order of try_2 his_2 tyr_1
Cross: try_2^+ his_2^+ tyr_1^+ —× try_2^- his_2^- tyr_1^-
SB 19 —× SB 100

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
try+	...	...	...	2600	82	610	4900
his+	...	418	2120	...	...	570	4360
tyr+	685	...	1540	...	25	...	2680

Transformant classes based on order: try_2 his_2 tyr_1.

TABLE 9

Linkage order of aro_2 try_8 his_2
Cross: aro_2^+ try_8^+ his_2^+ —× aro_2^- try_8^- his_2^-
SB 19 —× SB 200

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
try+	...	2670	6870	...	...	13950	14700
aro+	...	...	...	6830	1085	9630	13500
his+	9460	...	2040	...	430	...	9570

Transformant classes based on order: aro_2 try_8 his_2.

TABLE 10

Linkage order of aro_3 try_2 his_2
Cross: aro_3^+ try_2^+ his_2^+ —× aro_3^- try_2^- his_2^-
SB 19 —× SB 224

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
aro+	...	...	...	490	69	293	298
try+	...	154	251	...	...	251	485
his+	342	...	195	...	31	...	380

Transformant classes based on order: aro_3 try_2 his_2.

TABLE 11

Linkage order of try_2 his_2 aro_1
Cross: try_2^+ his_2^+ aro_1^+ —× try_2^- his_2^- aro_1^-
SB 19 —× SB 235

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
his+	...	189	610	...	...	436	858
aro+	545	...	322	...	70	...	1022
try+	...	...	...	260	60	104	882

Transformant classes based on order: try_2 his_2 aro_1.

TABLE 12

Linkage order of aro_2 try_2 his_2
Cross: aro_2^+ try_2^+ his_2^+ —× aro_2^- try_2^- his_2^-
SB 19 —× SB 190

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
aro+	...	...	...	4830	570	2165	5150
try+	...	1340	2240	...	...	2040	4330
his+	2110	...	2520	...	330	...	3300

Transformant classes based on order: aro_2 try_2 his_2.

The tyr_1-aro_1 sequence was studied further in the cross tyr_1^- ——× his_2^- aro_1^- (Table 13). Among the recombinants, his_2^+ tyr_1^- aro_1^-(110) could not be phenotypically distinguished from his_2^+ tyr_1^+ aro_1^-(100). A convenient progeny test was therefore devised on the expectation that tyr_1^- aro_1^+ ——× tyr_1^+ aro_1^- would give prototrophic recombinants whereas tyr_1^- aro_1^+ ——× tyr_1^- aro_1^- would not. After purification, the transformant clones were grown to competency, and a dropful of the culture was placed on a plate of minimal medium. After the spot had dried, a drop of his_2^- tyr_1^- DNA (approximately 5 μg) was placed on it. After incubation (40 hours) the appearance of prototrophic transformants was noted (Figure 4), and the frequencies of the 110 and the 100 classes estimated. The numerical data of Table 13 clearly indicate the order his_2 tyr_1 aro_1.

Four-point cross: The four linked markers: aro_2 try_2 his_2 tyr_1 can be scored independently. A four-point linkage test (Table 14) concurred with the three-point tests discussed so far.

Linkage of distal markers: To verify that a single molecule of DNA can indeed carry all of the markers of the linkage group, a doubly auxotrophic strain (SB 401) carrying the two distal markers was transformed with wild-type DNA and the cotransfer index determined (Table 15). The cotransfer index, 0.13, indicates that the same DNA molecule can carry all of the loci in this linkage group. Further, this cotransfer value is the lowest for any markers in this cluster

TABLE 13

Linkage order of his_2 tyr_1 aro_1
Cross: his_2^+ try_1^- aro_1^+ —× his_2^- tyr_1^+ aro_1^-
SB 103 —× SB 130

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
aro+	8000	...	2670	...	533*	...	13900
his+	...	...	...	1460	386	1670	11000

The competency procedure employed CHT–1 and CHT–10 supplemented with aromatic supplement. Transformant classes based on order: his_2 tyr_1 aro_1.

* These counts are based on purified transformant clones and were checked for prototrophy by platings on D.

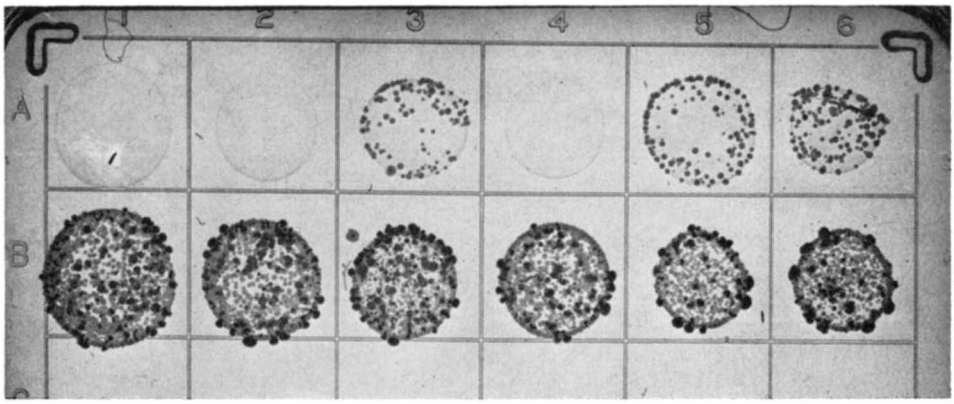

FIGURE 4.—Spot tests of ambiguous *tyr*± *aro*⁻ genotypes. The six *his*+ *tyr*± *aro*⁻ brought to competency by the yeast extract procedure. At 90 minutes, a drop of the culture, and after drying, a drop of DNA placed on top. Plate incubated for 36 hours at 37°C. Row A: his_2^- tyr_1^- aro_1^+ — × his_2^+ *tyr*± *aro*⁻. Row B: *his*+ tyr_1^+ aro_1^+ (SB 19) — × his_2^+ *tyr*± *aro*⁻.

TABLE 14

Four-factor cross

Cross: aro_2^+ try_2^+ his_2^+ tyr_1^+ —× aro_2^- try_2^- his_2^- tyr_1^-
SB 19 —× SB 202

Primary selection	Transformant class (per 0.1 ml of recipient culture)															
	0001	0010	0011	0100	0101	0110	0111	0101	1000	1001	1010	1011	1100	1101	1110	1111
try+	...	...	...	115	...	29	218	5.7	...	...	...	...	258	23	34	460
his+	...	46	189	...	...	20	240	...	...	...	5.5	31	...	...	36	460
tyr+	100	...	198	...	4.4	...	185	...	...	44	...	26	...	8.8	...	304
aro+	...	...	...	...	...	...	...	...	467	12	12	42	122	24	60	467

Transformant classes based on order: aro_2 try_2 his_2 tyr_1. DNA concentration—0.02 μg per ml of recipient culture. The culture brought to competence with CHT–2 and CHT–10 regimen supplemented with aromatic mix. See text for the method for estimating the number of transformants in each class.

TABLE 15

Cotransfer of distal markers

DNA source	Recipient cells	Cotransfer index
SB 19	SB 401	0.14
SB 188 + SB 279	SB 401	0.001

Total DNA concentration was 0.02 μg per 5×10^8 recipient cells. The cells were prepared for competency with CHT–2 and CHT–10, supplemented with the aromatic mix and 10 μg per ml of shikimic acid. The cotransfer index was calculated from the number of double and single transformant colonies assayed on appropriately supplemented media.

and supports previous evidence that these are the distal markers of those discussed so far.

Production of linked mutants with HNO_2: Additional loci linked to his_2 are systematically being sought. Nitrous acid treated SB 19 DNA has been used to transform his_2 to prototrophy on a medium complete for all nutrients except

histidine (ANAGNOSTOPOULOS and CRAWFORD 1961; NESTER and LEDERBERG 1961). Among the his_2 transformants, mutants of the following phenotypes have been recovered: *try*, *tyr*, *aro*, *leu*, *ade*, *ura*, and *met*. Only the first three are linked to his_2.

Biochemical studies: A summary of the nutritional responses of the aromatic mutants is given in Table 2. Some fragmentary additional information is now presented on the accumulation of intermediate metabolites of aromatic acid biosynthesis.

Multiple auxotrophs responding to shikimic acid: On a genetic basis these mutants fall into two classes—linked and not linked to his_2. These two classes have not yet been distinguished on a biochemical level. The difference between aro_2 and aro_3, two loci linked to his_2, is problematical and may only reflect the degree of completeness of the metabolic block. The two strains show different growth responses. aro_2 grows well on a combination of the five aromatic supplements, tyrosine, phenylalanine, tryptophan, p-hydroxybenzoic acid and p-aminobenzoic acid, whereas aro_3 requires 0.1 μg per ml of shikimic acid in addition to achieve good growth. Both mutants accumulate compounds in their filtrates which serve as a growth factor(s) for *E. coli* 83–1 under conditions of autoclaving which completely destroy 5-dehydroshikimic acid. In numerous instances, the growth promoting activity of filtrates of aro_3 was markedly increased (three- to ten-fold) after autoclaving, suggesting the accumulation of a phosphorylated intermediate. A compound having a bioautographic R_f similar to shikimic acid accumulated in filtrates of aro_2.

Multiple aromatic auxotrophs not fed by shikimic acid: Bioautography of the culture filtrate of aro_1 revealed a single spot having the R_f of shikimic acid, using *E. coli* 83–1 as the indicator mutant. This mutant feeds aro_2, and itself is fed by *E. coli* strain W7 (which accumulates prephenic acid). This supports the nutritional data that aro_1 follows aro_2 in the biosynthetic sequence. No studies were made on the nutritionally similar mutants which are not linked to the genes of aromatic synthesis.

Tyrosine mutants: The tyrosine mutants accumulate prephenic acid in their culture filtrates and grow on p-hydroxyphenylpyruvic acid, suggesting that the enzymatic block involves prephenic dehydrogenase. The possibility that *B. subtilis* might synthesize tyrosine from phenylalanine was studied by growing SB 112 (phe_1^-) with L-phenylalanine-1-C^{14}. The total cellular protein was isolated, hydrolyzed and the radioactivity of every amino acid (except tryptophan) determined. Only the phenylalanine was radioactive. The assay conditions were sensitive enough to detect the transfer of one percent of the radioactivity of the isolated phenylalanine to any other amino acid. In concordance with this datum, the twelve recombinable *tyr* mutants in our collection (which all map at the same locus) grow on p-hydroxyphenylpyruvic acid, but not on phenylalanine.

Phenylalanine mutants: phe_1 accumulates prephenic acid, and grows on phenylpyruvic acid, but not on tyrosine. When SB 65 (tyr_1^-) was cultured on L-tyrosine-3-C^{14} no radioactivity was observed in the isolated phenylalanine.

Additional loci linked to the try$_2$ *cluster:* The loci described in the preceding sections appear to have functions related to the structural control of biosynthetic enzymes. In addition to these loci, three other sites linked to the *try*$_2$ cluster may relate to the functional control of these enzymes.

Inhibition by histidine and phenylalanine: The strains carrying this mutant locus termed *inh*, grow very slowly if at all on media containing either L-histidine or L-phenylalanine. However, tyrosine, and its precursor, p-hydroxyphenylpyruvic acid antagonize this inhibitory effect. These effects are specific in that of all the amino acids only L-histidine and L-phenylalanine inhibit this strain. No other amino acid, or aromatic precursor tested (shikimic acid, anthranilic acid, indole, phenylpyruvic acid) or Mn^{++}, Mg^{++} or Cu^{++} reverses this effect.

A number of inhibition-resistant mutants have been isolated in a stock originally sensitive to histidine or phenylalanine. In most cases, but not all, mutants selected for resistance to histidine are also resistant to phenylalanine and vice versa. Two other independent and recombinable *inh*$^-$ mutants have been isolated which show qualitatively similar, but quantitatively dissimilar inhibitions by histidine and phenylalanine (SB 443 and SB 454).

Mapping studies on the inh *locus:* The position of this locus relative to the *tyr*$_1$ and *aro*$_1$ sites was mapped by the following cross: *tyr*$_1^+$ *inh*$^+$ *aro*$_1^+$ ——× *tyr*$_1^-$ *inh*$^-$ *aro*$_1^-$ (Table 16). *aro*$^+$ transformants were selected on tyrosine supplemented media, and 198 colonies analyzed for the remainder of their genotype. All colonies analyzed as *tyr*$^-$ were reverted to *tyr*$^+$ in order to permit an analysis of the sensitivity locus as either *inh*$^+$ or *inh*$^-$. This technique allows all four possible *aro*$^+$ transformant classes to be analyzed. On the expectation that the quadruple crossover class is the least frequent, the most likely order of those testable is *tyr*$_1$ *inh aro*$_1$. This order concurs with the cotransfer value for *tyr*$_1$-*aro*$_1$ determined previously (See Table 11). However, because the frequency of the class *tyr*$_1^+$ *aro*$_1^-$ *inh*$^-$ cannot be estimated in this cross, the possibility that the order may be *tyr*$_1^+$ *aro*$_1^-$ *inh*$^-$ cannot be ruled out. An additional cross was performed: *inh*$^-$ *tyr*$_1^+$ *his*$_2^+$ (SB 419)——× *inh*$^+$ *tyr*$_1^-$ *his*$_2^-$ (SB 70). Since 20 percent of the total transformants to either histidine or tyrosine were prototrophic, the order *his inh tyr* is unlikely. The *inh* loci of SB 126 and SB 128 (two other *inh* mutants isolated) map in the same general area as the *inh* locus of SB 419. A histidine-phenylalanine resistant revertant was mapped, and proved to be unlinked to the *try*$_2$ cluster.

TABLE 16

Linkage order of tyr$_1$ inh$_1$ aro$_1$
Cross: *tyr*$_1^+$ *inh*$^-$ *aro*$_1^+$ —× *tyr*$_1^-$ *inh*$^+$ *aro*$_1^-$
SB 419 —× SB 227

	Transformant classes (per 198 colonies)						
Primary selection	001	010	011	100	101	110	111
aro$^+$	23	...	21	...	11	...	143

Transformant classes based on order: *tyr*$_1$ *inh*$^-$ *aro*$_1$. *aro*$^+$ transformants were selected on S + tyrosine. 198 transformants were picked to N.A. and the remainder of their genotype determined by replica plating.

5-Methyltryptophan resistance: The growth of wild-type *B. subtilis* is inhibited by 5-methyltryptophan (Figure 5). However, it is possible to obtain spontaneous mutants resistant to the action of this analogue. Three independent spontaneous mutants resistant to one mg per ml were selected on gradient plates of minimal medium containing analogue.

Mapping studies of 5-mtr *resistance:* The marker of *mtr* and the try_2 locus are cotransferred with a frequency of 50 percent. The *mtr* locus was mapped more precisely by the following cross: $mtr^r\ aro_3^+\ tyr_1^+ \text{——}\times mtr^s\ aro_3^-\ tyr_1^-$ (Table 17). On the expectation that the quadruple crossover class (101) is the least frequent, the probable order is *mtr aro*$_3$ *tyr*. Thus the *mtr* locus is the distal marker at one end of the presently mapped try_2 segment. The two other mtr^r mutants isolated also map at this locus.

Site of action of 5-methyltryptophan: In *E. coli*, 5-methyltryptophan inhibits the formation of tryptophan, but its exact influence on the biosynthetic sequence is still under discussion. A resistant mutant secured in one step (but not genetically analyzed) is both derepressed in enzyme synthesis and resistant to the "feed back" inhibition of anthranilic synthesis by the analogue as well as by tryptophan (MOYED 1960). A 5-methyltryptophan resistant mutant isolated by COHEN and JACOB (1959) is insensitive to repression by tryptophan for the enzymes of tryptophan synthesis (tryptophan synthetase) and anthranilic acid synthesis.

In *Bacillus subtilis*, tryptophan, but not indole or anthranilic acid, will reverse the action of 5-methyltryptophan suggesting that tryptophan synthetase is the critical point of action. Some experiments on the conditions of formation of tryptophan synthetase are summarized in Table 18. The wild-type strain forms relatively little enzyme, presumably owing to repression by endogenous tryptophan; the level is reduced even further when tryptophan is added. The mtr^r mutant forms much higher levels, which are independent of the level of exogenous tryptophan, and the presence or absence of the try_2 mutation. These findings suggest that the effect of the mutation is to release the repression of tryptophan synthesis by tryptophan or its analogue.

Suppressor mutation: (*suh*). This strain has the unusual property of growing very slowly on S, but normally on *either* histidine or phenylalanine. To test whether this strain still carries the original his_2^- genotype, the following cross was carried out: SB 428 ——× 168, with try_2^+ transformants selected on minimal

TABLE 17

Linkage order of mtr aro$_3$ tyr$_1$
Cross: $mtr^r\ aro_3 + tyr_1 + \text{—}\times mtr^s\ aro_3^-\ tyr_1^-$

	Transformant class (per 0.1 ml of recipient culture)						
Primary selection	001	010	011	100	101	110	111
aro	...	250	324	...	...	255	330
tyr	340	...	...	...	<5	...	697

Transformant classes based on order: *mtr aro*$_3$ *tyr*$_1$.

TABLE 18

Tryptophan synthetase activity in 5-methyltryptophan sensitive and resistant strains

Strain no.	Genotype	L-tryptophan supplementation μg per ml	Units of tryptophan synthetase per 10^9 viable cells
SB 19	*mtr*^{s}	0	0.5
		20	0
SB 426	*mtr*^{r}	0	6.3
		20	6.3
168	*try*$_2^-$ *mtr*^{s}	1	3.0
		20	0
SB 474	*try*$_2^-$ *mtr*^{r}	1	6.4
		20	6.4

The strains were grown in S medium, containing the tryptophan supplement indicated to a level of 5×10^8 to 10^9 cells per ml. In addition, each culture contained 20 μg per ml of lysine, arginine, methionine, cystine, leucine, isoleucine, valine, phenylalanine, tyrosine, histidine, threonine, glutamic caid, proline, aspartic acid, alanine, glycine, serine and hydroxyproline.—Enzyme units correspond to 1 μM of indole disappearing per minute per 10^9 viable cells.

medium supplemented with histidine, phenylalanine, and histidine + phenylalanine (Table 19). Three phenotypes could be distinguished after replica plating to appropriately supplemented media: *his*$^+$, *his*$^-$, which responds to histidine or phenylalanine, and *his*$^-$ which responds only to histidine. Since a *his*$^-$ requirement is transferred with the *try*$_2^+$ at a high frequency, the *his*$_2^-$ locus is most likely still present in SB 428. These data further show that the ability of *his*$_2^-$ to grow on phenylalanine is conferred by a locus closely linked to the *his*$_2^-$ locus, the ability to grow on phenylanlanine being cotransferred with the *try*$_2$ locus with a minimum frequency of 65 percent.

DISCUSSION

The genetic map of the transforming DNA molecule which carries many of the *aro* loci in *B. subtilis* (the *try*$_2$ segment) is given in Figure 6. The tryptophan operon can now be viewed as part of an even larger ensemble related in common

TABLE 19

Linkage of suppressor locus to try$_2$
Cross: *try*$_2^+$ *his*$_2^\pm$ *suh*$^-$ —× *try*$_2^-$ *his*$_2^+$ *suh*+

Medium supplementation for primary selection	Additional growth response of transformants		
	1* *his*$^+$ *suh*$^\pm$	2† *his*$^-$ *suh*$^-$	3‡ *his*$^-$ *suh*+
histidine	16	71	13
phenylalanine	35	65	0
histidine + phenylalanine	15	82	3

DNA, isolated from SB 428, was used to transform 168 recipient cells. The transformants were plated on three different media (primary selection). One hundred colonies from each medium were stroked onto nutrient agar and replica plated to appropriate medium to give the growth responses, 1, 2, and 3. The figures in the table refer to the number of colonies of the 100 picked from the primary selection medium, which fall into the particular growth response category. No colonies were capable of growing on phenylalanine but not histidine. The wild-type strain is designated as *suh*$^+$; the mutant which manifests the suppression as *suh*$^-$. Because of our limited knowledge of the phenotypic expression of the suppressor, we have no basis for deciding on *suh*$^+$ or *suh*$^-$ for growth-response 1.

* Growth on D (as well as histidine and phenylalanine supplemented plates).
† Growth on D + histidine and D + phenylalanine, but not D.
‡ Growth on D + histidine, but not D + phenylalanine.

to the biosynthesis of aromatic amino acids. Further studies on the regulation of the various enzymes are now needed to verify the hint that these clusters do constitute one or more operons (JACOB and MONOD 1961).

The present level of genetic and especially of biochemical information about the mutants does not yet permit a critical alignment of the sequence of biosynthetic functions and the corresponding loci in the try_2 cluster. In addition, some mutants (aro_4, aro_5) with enzymatic defects in closely related biosynthetic reactions are unlinked to each other or to the try_2 cluster.

The present statement of the pathway of *aro* biosynthesis in *B. subtilis* is derived primarily from the nutritional requirements and accumulations of the various *aro* mutants and corresponds to the general pattern observed in *E. coli*. In particular, shikimic acid appears to be an intermediate, although the stage in the pathway at which phosphorylation occurs is not clear. Further, as in *E. coli* prephenic acid appears to be the last common intermediate of tyrosine and phenylalanine biosynthesis; phenylalanine is not a direct precursor of tyrosine.

The interpretation of the inh^- mutation is obscure. The *inh* locus may be part of the structural gene involving tyrosine biosynthesis since it maps very close to it. This suggests an altered enzyme inhibited by histidine and phenylalanine or perhaps more plausible the repression of its synthesis. Since tyrosine and p-hydroxyphenylpyruvic acid, but not phenylalanine or tryptophan, overcome the inhibition, an enzyme of tryosine biosynthesis after prephenic acid presumably prephenic dehydrogenase is most likely involved. Attempts to detect the accumulation of prephenic acid under conditions of histidine inhibition have thus far been negative.

Histidine is related to aromatic biosynthesis, or more likely its regulation, as evidenced in several ways whose significance is as yet obscure: (1) the mapping of the his_2 locus in the try_2 cluster; (2) the interchangeability of histidine and phenylalanine in feeding suppressed his_2 mutants; (3) the inhibition of growth of inh^- strains by histidine, relieved by tyrosine; (4) the resistance of his_2 to inhibition by 5-methyltryptophan (Figure 5). These findings reinforce a suspicion that his_2 is not a structural gene for an enzyme of histidine biosynthesis but that a histidine requirement is established as a byproduct of deviations in the regulatory systems of aromatic biosynthesis.

The present map extends the estimated number of distinct genes which can be carried on a single molecule of transforming DNA to 13. This estimate makes several assumptions: (1) the number of enzymes involved in tryptophan biosynthesis is the same in *B. subtilis* and *E. coli*; (2) aro_3 and aro_2 are distinct loci; and (3) the *inh*, *mtr* and *suh* genes are not allelic to any of the structural genes described. The application of some contemporary theory might allow for as many as 20 genes on such a molecule.

Sedimentation analysis of *B. subtilis* DNA indicates a characteristic molecular weight in the range of 10 to 15×10^6, corresponding to approximately 20,000 nucleotide pairs (NESTER, GANESAN and LEDERBERG 1962). A coding ratio of three nucleotide pairs for one amino acid would require only 1,000 nucleotide pairs to specify one protein sequence of some 300 amino acids (the size of the A protein of tryptophan synthetase in *E. coli*, [HENNING, HELINSKI, CHAO and

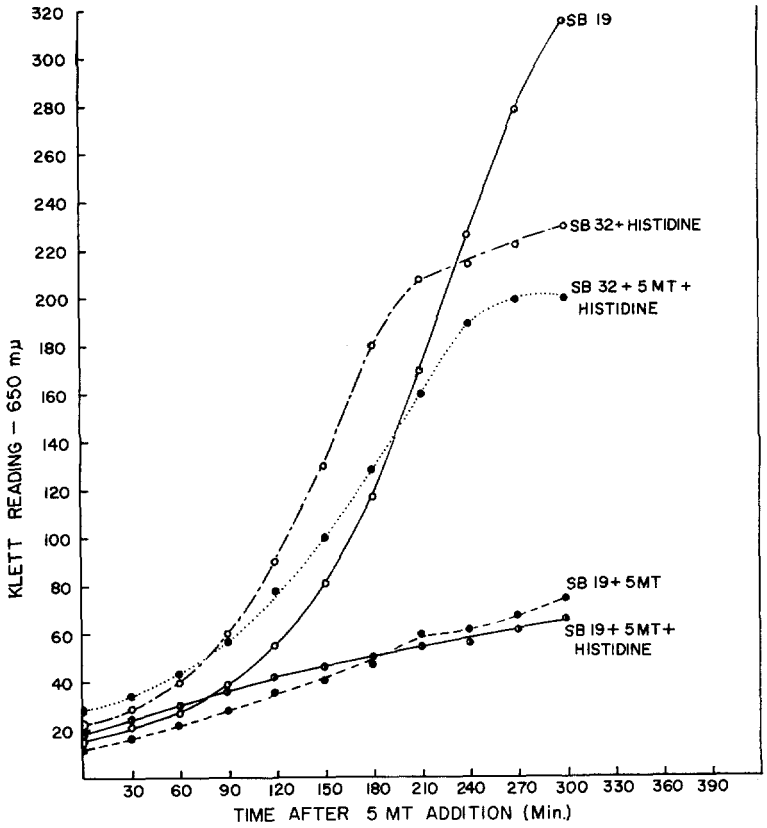

FIGURE 5.—Inhibition of wild-type *B. subtilis* with 5-methyltryptophan (5 mt). SB 19 was grown 12 hours in S + 0.05 percent casein hydrolysate (acid-hydrolyzed), washed once, and 0.5 to 1 × 10^8 cells per ml inoculated into S medium in side-armed flasks. Cultures were incubated with aeration on a reciprocal shaker at 37°C for 120 minutes, at which time 50 μg per ml of 5-methyltryptophan (or an equal volume of water) was added. All media contained 0.01 percent casein hydrolysate (acid hydrolyzed). L-histidine was added at 10 μg per ml final concentration.

YANOFSKY 1962]), thus allowing for twenty genetic loci per DNA molecule. On this reasoning, over half of the loci on the molecule carrying the *aro* linkage group have been identified.

The linkage map (Figure 6) is quantitated on crossover frequency, on the assumption that the distance between two loci is inversely correlated with the frequency of their joint incorporation into the same transformed cell. The development of a sound metric for mapping must await a more detailed theory of crossing over. The separation of linked markers may occur at two stages: in the course of preparation of the DNA, and in stages following the addition of DNA to the recipient population. We have no positive evidence on the causes of such breakage. Shearing forces may break the DNA during its extraction or subsequent handling. Nucleases may also play some part in the disruption of DNA after its

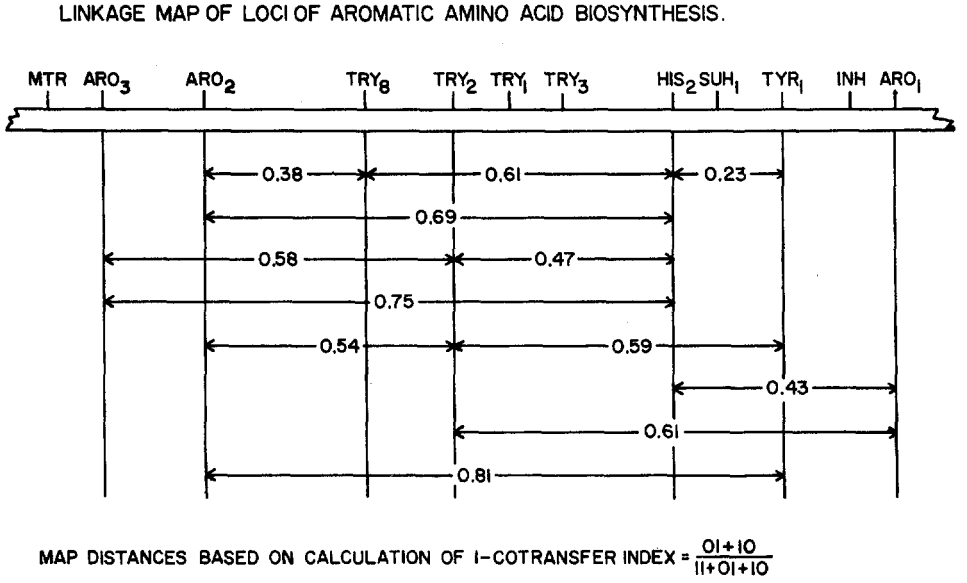

FIGURE 6.—Linkage map of loci of aromatic biosynthesis: All distances are calculated from the data of three-point crosses using the formula: $q = 1 - r = (10 + 01)/(10 + 01 + 11)$. The map location of the try_1 and try_3 loci was taken from the data of ANAGNOSTOPOULOS and CRAWFORD (1961). These loci are distinct from both try_2 and try_8. The reader is referred to their paper for the enzymatic reactions controlled by these loci.

uptake by the recipient bacterium. The order of loci based on the frequency of two-point transfer concurs with the sequence assigned by designating the least frequent class as the quadruple crossover class. However, since cotransfer values based on two-point transfers suffer from a lack of internal controls, and from difficulties of statistical estimation, any order of loci should be verified by three-point crosses. Not only do such crosses provide a qualitative proof of the order of the loci, but map distances can be assessed with a greater degree of certainty. No provision for possible polarity of transformation has been required for the interpretation of the four-point cross SB 19 ——× SB 202.

SUMMARY

The number of different genetic loci carried by a single transforming molecule of DNA in *B. subtilis* has been extended to a maximum of 13. These markers can be placed in a linear linkage map, with cotransfer indexes ranging from 0.77 for the his_2-tyr_1 markers to 0.13 for the aro_1-aro_3 markers. Every locus, except one involving a requirement for histidine, is involved in the biosynthesis of aromatic amino acids. Included in this cluster are genes controlling enzymes of shikimic acid, tryptophan, tyrosine and phenylalanine synthesis. However, several loci involved in shikimic acid, tyrosine and phenylalanine synthesis are neither linked to this cluster, nor linked to each other. Included in this operon are at least three genes which may regulate the synthesis of the structural enzymes. One

locus results in inhibition by histidine and pheylalanine, reversed by tyrosine; a second confers resistance to 5-methyltryptophan, and the third is a suppressor mutation which allows phenylalanine to replace histidine in the histidine requiring strain.

A preliminary analyses of the biochemical pathway of aromatic acid biosynthesis suggests that *B. subtilis* uses the same general pathway as *E. coli*.

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